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Studies with fluorescence-labeled antibodies. V. Simultaneous demonstration of several antigens by means of variously colored fluorescent antibodies, using Proteus morganii and Bacillus cereus as examples.

by G. Poetschke, H. Uehleke and L. Killisch.

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Recent developments of great promise which permit demonstration and microscopic localization of minute quantities of antigen by Coons' fluorescence-serological method were limited only by the fact that a single pigment (fluorescein isocyanate) was available for the production of antibody conjugates. This required that each antigen be demonstrated separately.

Simultaneous identification of two or more antigens in the same preparation necessitates the coupling of different antibodies to substances of varicolored fluorescence.

It is desirable that emission maxima of the contrasting pigments be separated sufficiently on the spectrum, while still being induced to fluoresce by the same excitor source and, if possible, being passed through the same ocular suppression filter.

1-Dimethylamino-naphthalene-5-sulfonic acid (Weber 1952, Clayton 1954, Laurence 1957, Mayersbach 1957, 1958, Petuely 1958, Redetzky 1958, Wolochow 1959) is not suitable for simultaneous application with fluorescein isocyanate. Both pigments fluoresce in the green zone.

We therefore searched for red, yellowish-red or yellow dyes. Independently of and simultaneously with Chadwick et al. (1958), Uehleke (1958 a and b) showed that various pigments that contain sulfonic acid groups may be linked to proteins as sulfonic acid chlorides and thus become feasible as optical tracer groups for antibodies. Uehleke has previously reported on the production and properties of such antibodies. Sulfo-rhodamine B, which fluoresces in the reddish-yellow zone, and 3-hydroxy-pyrene-5.8.10-trisulfonic acid, which gives off yellowish-green fluorescence, were chosen for additional tests because of their contrasting pigments. In the course of our investigations we learned of Silverstein's (1957) paper on varicolored labeling of antibodies. He utilized the isocyanates of fluorescein and rhodamine B.

We chose two morphologically dissimilar organisms as models for simultaneous demonstration of two different antigens: Proteus morganii and Bacillus cereus. We had previously subjected the Proteus strain and its labile and stable L-phase to fluorescent-serological study (Poetschke, Killisch, Uehleke, 1957).

Various authors have reported on identification of bacterial species and types with fluorescence-lableled antibodies. Malleomyces pseudomallei (Moody, Goldman and Thomason, 1956; Thomason, Moody and Goldman, 1956), S. typhosa (Thomason, Cherry and Moody, 1957; Silverstein, 1957), Streptococcus (Moody, Ellis and Updyke, 1958; Halperen, Donaldson and Sulkin, 1958), H. pertussis (de Repentigny and Frappier, 1956); several species from the bovine stomach (Hobson and Mann, 1957), E. coli (Petuely, 1958; Whitaker, Page, Stulberg and Zuelzer, 1958), Bc. anthracis (Levina, 1958), Shigella (LaBrec, Formal and Schneider, 1958; Kabanova and Glubokina, 1958), P. pestis (Carter and Leiss, 1958; Wolochow, 1959), P. tularense, Br. suis and V. comma (Carter and Leise, 1958), Pneumococcus (Silverstein, 1957).

The behavior of isolated bacterial antigens in the animal organism has been described by other authors: capsular polysaccharide of Pneumococcus (Coons, Creed, Jonas and Berliner, 1942; Kaplan, Coons and Deane, 1950), Kl. pneumoniae capsular polysaccharide, group A (Schmidt, 1952), Streptococcal M protein (Kaplan, 1958), Streptococcal hyaluronidase (Emmart, Cole, May and Longley, 1958).

Material and methods

Antigens: Proteus morganii, strain 18, Bacillus cereus, strain RK in agar or broth cultures, incubated at 37°C for 18-20 hours. Suspensions in NaCl or sterile nutrient broth were streaked on slides. Air-dried preparations were fixed at 37°C with methanol and subsequently dried at the same temperature.

Immune sera: Rabbits were immunized intravenously six times at intervals of 2-3 days with increasing doses of the repeatedly washed, killed bacterial suspensions. Native immune sera against Proteus agglutinized up to dilution 1:12800. In view of the spontaneous agglutination of Bc. cereus, the upper limit of agglutination could not be established precisely, but was found to be above 1:1000.

Gamma globulin was precipitated by addition of 40% (v/v) saturated ammonium sulfate solution. Precipitation was repeated once. Gamma globulin solutions were stored at -24°C after dialysis against NaCl - phosphate buffer solution (pH $\sim$ 7.4).

Preparation of sulfochlorides: The method described by Uehleke (1958 b) was slightly modified. Dry PCl_5 was placed in a dry mortar with the pigment (sulforhodamine B or 3-hydroxy-pyrene-5.8.10-tri-

sulfonic acid) in a ratio of 2:1. The mixture is vigorously ground for a few minutes under the aspirator. Resultant sulfochloride is dissolved in 10 ml of cold, non-aqueous acetone and filtered into a ground tube charged with some Na_2SO_4. In the case of pyrene sulfochloride, precipitation must be prevented by adding 0.5 to 1.0 g tri-(hydroxymethyl)-aminomethane (tris buffer) in substance to the acetone prior to filtration. Sulfochlorides, dissolved in acetone, are stored at ca. $+1^{\circ}C$ in the exsiccator, where they are preserved for some time.

The concentration of rhodamine-sulfochloride was established colorimetrically by diluting first with acetone (1:20) and then in water (terminal dilution 1:5000 to 1:10000 of the stored solution). As standard we used non-chlorinated pigment. Pyrene-sulfochloride is not suitable for such measurements.

Conjugation: 4-6 mg rhodamine-sulfochloride were added to 100 mg protein, determined by the biuret method. 2 ml protein solution, 4-6 ml 0.25 mole tris buffer, pH 9, and 10% of the total volume in dioxane are mixed in a wide test tube. All solutions must be pre-cooled to ca. $+1^{\circ}C$. Sulfochloride, diluted with acetone to a volume of about 0.5 ml, is added to the globulin solution in small amounts with a tuberculin syringe under constant stirring.

We added pyrene sulfochloride to the acetone solution in quantities that still showed dispersal without turbidness in the protein solution. Conjugates were stirred for 4 hours in the cold. This was followed by cold dialysis against repeatedly replaced NaCl-buffer solution, pH 7.4. Conjugated globulin solution was mixed once or twice with animal charcoal (50 mg/ml), allowed to stand for 15 minutes at room temperature after repeated stirring, and then centrifuged under cold conditions at ca. 10000 r.p.m. Treatment with liver powder proved unnecessary when sera of high quality were involved, since remnants of non-specific coloration disappear as soon as sera are diluted for staining (1:5 to 1:40). Conjugated sera were stored at $+4^{\circ}C$. We did not observe bacterial contamination of sera. It seems that conjugated sera had antibacterial properties.

Staining as described by Coons: Globulins subjected to varicolored conjugation may be employed singly in succession or as a mixture 1:1.

Fluorescence microscopy: Large fluorescence apparatus by C. Zeiss, Oberkochen, with HBO 200 Hg high pressure burner. Excitor filter: 1-2 BG 12 and one heat screen filter. Ocular screen filters OG 4 and OG 5 by C. Zeiss. The entire optics was a normal glass optics. A metal surface mirror and a condenser with high aperture are recommended.

Results

As shown by Fig. 1, both bacterial species are stained specifically by homologous immune sera. Anti-Proteus globulin colored green with hydroxy-pyrene trisulfonic acid chloride stains only Proteus organisms, which are distinctly different morphologically from Cereus rods or spores electively stained red with homologous immune globulin labeled with rhodamine. It did not matter whether globulins were employed separately and in succession, or mixed and simultaneously. Slides of E. coli did not show non-specific coloration. Staining with labeled globulin was specifically impaired by pre-treatment with homologous, unlabeled globulin.

Discussion

Results show that dyes with sulfochloride groups may be used in fluorescence-labeling of antibodies. Since stains with fluorescence of various colors are available, there is a possibility that two or more different antigens in the same preparation may be demonstrable in the future. The fact that both labeled antibodies may be used simultaneously as a mixture, without loss of staining specificity, is certainly a step forward. A number of authors found that the fluorescence-serological method offers valuable new possibilities for the identification of minute bacterial traces. We are only beginning to utilize this new tool in diagnosis and in pathogenetic, immunological as well as ecological work.

Although the simplicity inherent in the preparation of sulfochlorides is remarkable when compared to the effort required by isocyanates, we do not claim that these substances and the method are ideal. The methodology certainly needs improvement in several directions: The reaction during grinding of the sulfo dye with PCl_5 is not quantitative. Moreover, PCl_5, PCl_3 and $POCl_3$ are dissolved in acetone in addition to the pigment sulfochloride. In the described process, these remain in the dye-sulfochloride solution, a circumstance which seems to impair its stability. At any rate, they decompose acetone partially after prolonged standing, resulting in brown discoloration. Attempts to purify the material on the basis of the poor solubility of sulfochlorides in water were not satisfactory. Stain-globulin conjugates revealed quite variable durability. Rhodamine was most durable, while oxypyrene occasionally showed unsatisfactory stability. Protein may settle out even during conjugation. It remains to be clarified whether dyes having several sulfochloride groups are distributed on more than one protein molecule or protein-pigment complex. This situation may explain why conjugated sera show considerable losses in titer (up to 1/100). Only sera of very high quality should be conjugated. In the case of sulfo-rhodamine, especially, the relatively great stability of staining with respect to the bleaching effect of the excitor source must be stressed.

Red-fluorescent rhodamine seems to be worthy contribution to the labeling pigments available today. Hydroxy-pyrene-trisulfonic acid is less satisfactory and should be replaced by green fluorescein-isocyanate or dimethyl-amino-naphthalene-sulfochloride, since these substances are now available commercially.

We are unable to make comparisons with the contrasting isocyanates used by Silverstein (1958), since we did not employ them.

Summary

1. The labeling of immune gamma globulins with the sulfochlorides of various fluorescent dyes is described.

2. Sulfo-rhodamine B, a pigment with reddish-yellow fluorescence, proved to be a feasible contrasting agent for hydroxy-pyrene-trisulfonic acid chloride and other green-fluorescent labeling substances.

3. P. morganii and Bc. cereus were used to demonstrate that simultaneous specific staining with a mixture of variously labeled antibodies is possible.

4. The potential of the method, ~~as well~~ an as its advantages and disadvantages, are discussed.

Illustration

Plate III. B. cereus, stained with anti-cereus globulin, labeled with rhodamine B-sulfochloride (red). Proteus morganii, stained with anti-Proteus globulin, labeled with 3-hydroxy-pyrene-5.8.10-trisulfochloride (yellowish-green).